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(57) Abstract

The invention relates to a new use of a known chemical compound as a therapeutic agent having antiarrhythmic properties, the ceruloplasmin (a copper protein). More particularly, the Applicants have noted that ceruloplasmin generates antiarrhythmic effects during reperfusion in the ischemic heart. The invention also relates to a method for either preventing or treating heart arrhythmias.

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ANTIFIBRILLATORY AGENT IN MYOCARDIAL REPERFUSION

Field of the invention

The present invention relates to a new use of a known compound as a therapeutic agent having antiarrhythmic properties, the ceruloplasmin (a copper protein). More particularly, the Applicants have noted that ceruloplasmin generates antiarrhythmic effects during reperfusion in the ischemic heart. The invention also relates to a method for either preventing or treating heart arrhythmias.

Background of the invention

Over the last decade evidence has been accumulated that oxygen free radicals (OFR) are, at least in part, responsible for specific damages and arrhythmias at reperfusion of ischemic heart. Various pathways generating superoxide radical ($\cdot O_2^-$) and other reactive oxygen intermediates (ROI) have been identified, such as: activation of polymorphonuclear leukocytes, autoxidation of catecholamines, reactions of xanthine oxidase and NADPH oxidase, or the arachidonic acid metabolism. The harmfull effects of superoxide radical and its by-products are dramatically increased in the presence of transition metals. The ferrous (Fe2+) ion generated by Haber-Weiss reaction catalyses the formation of the highly aggressive hydroxyl ($\cdot OH$) radical, via the Fenton reaction. The presence of free radicals has been already measured in ischemic and reperfused myocardium directly by electron paramagnetic resonance spectroscopy and indirectly by biochemical assays of malondialdehyde (MDA) as indicator for lipid peroxidation. The OFR concentration at reperfusion is higher than during ischemia. Free radicals may contribute to reperfusion injury by interacting with membrane polyunsatured fatty acids (PUFA) and generating lipid peroxides which increase th membranes permeability and

alter ionic homeostasis. Lipid peroxidation of myocardial membranes by OFR, has been considered a potential mechanism of reperfusion arrhythmias. Inhibition of free radical accumulation during myocardial ischemia and reperfusion with OFR scavengers, antioxidant enzymes, and spin-trap agents was shown to reduce the severity of reperfusion-induced arrhythmias in many studies.

Ceruloplasmin, the blue copper plasma oxidase (EC 1.16.3.1) is a multifunctional protein (a2 -globulin) involved in copper transport and control of the level of serum biogenic amines. Ceruloplasmin, known also as ferroxidase I, catalyses the oxidation of Fe2+ ---> Fe3+ (25), reducing thus the level of Fe2+ available to produce hydroxyl radicals via Fe2+ dependent radical forming Fenton and Haber-Weiss reactions.

Ceruloplasmin, also considered the most prominent serum antioxidant, was shown to scavenge a variety of OFR in vitro. The activity of ceruloplasmin was found to be several times lower in experimental animals as well as in patients with severe ischemic heart disease, or with acute infarction.

Recently, ceruloplasmin has been shown to exhibit protective effects against OFR generated by electrolysis in the coronary arterial perfusate on isolated rat heart. Ceruloplasmin prevented the production of malondialdehyde and restored the levels of myocardial creatine kinase, aspartate transaminase, lactate dehydrogenase, calcium and noradrenaline.

In the same conditions, the Superoxide dismutase (SOD) was unable to prevent the damages generated by oxidant species induced by electrolysis with the same effectiveness as ceruloplasmin. However, the SOD afford a good antiarrhythmic

protection at reperfusion of the ischemic heart.

Therefore, it would be highly desirable to obtain a therapeutic agent which protects heart against oxidants species associated various types of oxidative stress and at the same time, to present antifibrillatory effects in arrhythmias associated with the reperfusion of ischemic hearts. Such therapeutic agent will be of a high utility, since it was recently shown that fibrillation generates OFR (P. Ferdinandy, D. K. Das, A. Tosaki - J.Mol. Cell. Cardiol. 25, 683-692, 1993).

Summary of the invention

The present invention relates to a new use of ceruloplasmin or pharmaceutically acceptable derivative of ceruloplasmin, as an agent for protecting an organ from oxidative stress, and preferably as antifibrillatory agent against heart arrhythmias.

Advantageously, the ceruloplasmin shows a spectral parameters ratio ($Absorption_{610}/Absorption_{280}$) greater than 0.04, preferably of at least 0.06.

Advantageously, the ceruloplasmin may be obtained by a process involving the following sequence of essential steps:

- fractional precipitation of plasma proteins;
- recovering of the supernatant;
- adsorbing the proteins still contained in the supernatant on a chromatographic support comprising an aminoethyl resin; and
- desorbing the proteins adsorbed on the chromatographic support with an appropriate eluant and recovering a chromatographic fraction containing ceruloplasmin having a spectral parameters ratio ($Absorption_{610}/Absorption_{280}$) greater than 0.04. Preferably, the ceruloplasmin has a

spectral parameters ratio ($Absorption_{610}/Absorption_{280}$) of at least 0.06.

Optionnally, the proteins obtained may be further subjected to an ultrafiltration step.

Advantageously, the fraction of protein containing the ceruloplasmin having a spectral parameters ratio ($Absorption_{610}/Absorption_{280}$) greater than 0.04, preferably 0.06, is further associated with a pharmaceutically acceptable excipient to define a pharmaceutically acceptable composition.

Advantageously, the pharmaceutically acceptable excipient may be of the type commonly used for the preparation of a composition for an intravenous injection.

Advantageously, the composition may have a ceruloplasmin concentration of at least 0.50μM, preferably of at least 1μM.

The invention also relates to a pharmaceutical composition for protecting an organ from an oxidative stress, wherein it comprises in association with a pharmaceutically acceptable carrier or a ceruloplasmin, a pharmaceutically acceptable ceruloplasmin derivative.

Preferably, the invention relates to an antifibrillatory pharmaceutical composition against heart arrhythmias, wherein it comprises in association with a pharmaceutically acceptable carrier or a ceruloplasmin, pharmaceutically acceptable derivative of ceruloplasmin.

More particularly, the invention relates to an antifibrillatory composition wherein the ceruloplasmin has the aforesaid preferred characteristics.

The invention also relates to a method for protecting and organ from an oxidative stress wherein a composition as defined in hereinbefore is administered to said organ, and especially a method for treating or preventing heart arrhythmias.

In accordance with a particularly preferred embodiment of the present invention, there is provided a preparation of therapeutic agent made up with ceruloplasmin purified from bovine plasma by chromatography on Aminoethyl (AE)-Agarose, following a recently described method.

The protective effect of Ceruloplasmin (CP) against reperfusion induced arrhythmias has been investigated on partial (15 min) and total (30-45 min) ischaemic isolated rat heart.

Partial ischeamia was induced by occlusion of left descending artery, followed by 10 min reperfusion. Left ventricular pressure and epicardial ECG were continuously monitored before and during ischaemia and reperfusion. A control group was submitted to partial ischaemia without CP treatment (n = 12). Dose-effect relationship and the role of CP molecular integrity in cardioprotection were established by treatment of ischemic hearts with different concentrations of ceruloplasmin (0.25 μM, 0.50 μM, 1 μM, 2 μM) and at three different degrees of molecular integrity (A610/A280 = 0.02; 0.04; 0.06).

Electrophoretically homogeneous CP (1 μM), was injected (0.5 mL/min) in the Krebs-Henseleit perfusing buffer (15 mL/min) in the inflow cannula, above the heart. Another groups of hearts were treated throughout the experiment with boiled d natured ceruloplasmin (1μM). For comparison, we have examined the antiarrhythmic effects of deferoxamine (500 μM) - an iron chelator produced by bacteria (Streptomices

pilosus).

The incidence of reversible and irreversible ventricular fibrillations were significantly lower in the ceruloplasmin treated groups, in dose and molecular integrity dependent manner.

The incidence of irreversible ventricular fibrilation was significantly diminished from 89% (control group) to 28 % (CP treatment group). In total ischemia, CP caused a significantly decrease of premature ventricular beats (PVB) duration from 3 - 4 min (control) to 0.5 - 1 min (CP treatment).

Deferoxamine (500 μM) reduced the incidence of ventricular fibrillation to the same degree as ceruloplasmin (1 μM) but at concentration 500 times higher. Ceruloplasmin seems to protect ischaemic heart against reperfusion induced arrhythmias, exerting a strong antifibrillatory effect at reperfusion . The protective effects of ceruloplasmin are highly related to its molecular conformation and integrity.

In the tables

Table I illustrates the ceruloplasmin dose-effect dependency of the reperfusion-induced arrhythmias, in conditions described in this invention;

Table II shows the dependency of cardioprotection on the molecular integrity of the ceruloplasmin, in conditions described in this invention;

Table III presents comparative antiarrhythmic effects of ceruloplasmin and deferoxamine in conditions described in this invention.

Detailed description of the invention

The data obtained in this invention clearly indicate the capacity of ceruloplasmin to reduce significantly reperfusion-induced ventricular fibrillation in isolated rat heart.

During early reperfusion of ischemic myocardium, the sudden influx of oxygen in presence of reduced metabolic intermediates accumulated during the ischemic period, will provide an ideal situation for the formation of OFR, exceeding the antioxidative capacity of the tissue. Oxygen free radicals, in particular the hydroxyl radical, may exacerbate ischemia induced injury by promoting oxidative modifications in cell membranes phospholipids, enzymes, and ionic pumps. Altered electrophysiological membrane activity and calcium overload has been suggested as important factors underlying OFR-induced reperfusion arrhythmias.

For the antifibrillatory effects of ceruloplasmin the Applicants supposed a mechanism related to its ferroxidase activity. Ceruloplasmin may decrease the amount of ferrous ion preventing thus the production of hydroxyl radical via the Fenton and Haber-Weiss reactions.

Mechanisms of iron involvement are not fully elucidated, but there is a growing consensus that oxidative tissue damage is related to non-heme cellular iron mobilized from cytosolic metal-containing sites: e.g. myoglobin, and ferritin stores within endothelial and myocardial cells. Most of intracellular iron is deposited in ferritin (which can store 2000 up to 4500 of Fe^{3+} ions per complex) from where in the presence of reducing equivalents (e.g. superoxide radical), is released in the ferrous (Fe^{2+}) form. This may explain the toxicity of superoxide anion. The initial damage results in a generalized release of iron into the cellular environment,

and more widespread nonspecific injury may result.

Considering the high molecular weight of ceruloplasmin (132 KDa), the protective intervention of ceruloplasmin is preferably for the vascular space. Thus, superoxide anion produced in endothelial cells at reperfusion generates hydroxyl radicals via the iron-catalyzed Fenton reaction, damaging in this way the endothelium and adjacent contractile or conducting cells. For extracelluar ceruloplasmin to be effective as a ferroxidase in the case of intracellular OFR production, one should assume the outside diffusion of ferrous ions and of superoxide radicals. Both superoxide anion and hydrogen peroxide have longer half-lives than the hydroxyl radical and can readily permeate cell membranes, either directly (H_2O_2) or through anion channels (superoxide radical). It is known that iron salts can penetrate a membrane in micromolar range concentrations. In this way, ceruloplasmin might be able to exert its ferroxidase activity and prevent hydroxyl radical formation from an intracellular source of superoxide radicals.

On the other hand, the protection of myocardium against intracellular OFR can hypothetically be explained by transcytosis of ceruloplasmin from coronary capillaries into myocytes. A similar process has been already put in evidence for exogenous superoxide dismutase and catalase which, after a brief episode of regional ischemia , were concentrated and transported across the capillary endothelium and into myocytes. In fact the presence of specific receptors for ceruloplasmin in aortic cell membranes and in heart tissue has been evidenced.

Alternatively, the beneficial effects of ceruloplasmin might be due to the prevention of hydroxyl radical generation from an extracelluar source of superoxide production. In the model of isolated heart , the only extracelluar source of OFR

production could be the autoxidation of cathecolamines released from nerve endings, which accumulate in abnormally high concentrations in the ischemic myocardium. It is known that ceruloplasmin significantly reduces the increase of noradrenaline efflux in the perfusate after electrolysis of perfusing buffer in isolated heart, suggesting a protection against free radical-induced injury to the sympathetic nerve endings.

The protective antiarrhythmic effects of ceruloplasmin are dose-dependent, in the range 0.5 μM - 2.0 μM. Protection is exerted against total ventricular fibrillation, especially against irreversible ventricular fibrillation and the normal sinus rhythm duration (survival time) which is significantly increased. Ceruloplasmin at a dose higher than 1μM is not significantly more effective on the incidence of ventricular arrhythmias and on the duration of normal sinus rhythm. This fact suggests that ceruloplasmin acts catalytically as ferroxidase rather than stoichiometrically as a simple radical scavenger.

The molecular integrity of the ceruloplasmin blue-copper center, responsible for the A610 nm and important for the ferroxidase activity, appears to be an intrinsic condition for the antifibrillatory effects of ceruloplasmin.

Although the incidence of total ventricular fibrillation is significantly decreased at a molecular integrity coresponding a ratio A610/A280= 0.04, a significant reduction of irreversible ventricular fibrillation is noticed only at treatment with ceruloplasmin of a higher degree of molecular integrity (A610/A280 = 0.06) suggesting that the protective effects of ceruloplasmin are highly related to its molecular conformation.

The importance of tridimensional active conformation of

ceruloplasmin is strongly supported by the lack of protective effects of boiled ceruloplasmin. In fact, heat denaturation of ceruloplasmin induces a complete loss of enzyme activity, of blue color and of the blue-copper center integrity indicating dramatic changes in the protein conformation. Although ceruloplasmin and deferoxamine (iron-chelating agent), act by different mechanisms to reduce the availability of ferrous ions, their ultimate protective effects are probably exerted by the same prevention of hydroxyl radicals. In our study, deferoxamine (500 μM) presents a strong and more generalized antiarrhythmic effect but at a concentration much higher than ceruloplasmin (1 μM).

Therefore, ceruloplasmin exerts a strong antifibrillatory effect during reperfusion in the ischemic isolated rat heart justifying further consideration of ceruloplasmin as a powerful protective agent against irreversible ventricular fibrillation, the most severe type of reperfusion-induced arrhythmias.

Example 1

Ceruloplasmin preparation

The purification of ceruloplasmin (EC 1.16.3.1) leads to an electrophoretically homogeneous form and with specific activities higher than those obtained by previously described purification techniques. Two common steps: precipitation of bovine plasma proteins with ammonium sulphate (at 35 % and 55 % saturation) followed by column chromatography on AE-Agarose. Aminoethyl (AE)-Agarose is a chromatographic material (not commercially available), obtained in our laboratory by the treatment of agarose beads (Clsepharose 4B , Pharmacia, Uppsala, Sweeden) with 1-chloro-2-ethylamine (chlorohydrate) from Aldrich (Milwaukee, Wi), under conditions previously described. The resulting

chromatographic material contains amino-ethyl functional groups and is called AE-Agarose.

The chromatographic material was prepared in the following way. Sepharose 4B, 300ml gel bed suspended in 100ml of 5N NaOH, was optionnally previously treated with epiclorohydrine, 25 ml, at 70°C for two hours, and then, after exhaustive washing and resuspension in NaOH 10N, with 130 ml of 100% cloroethylamine at 70°C for two hours. In this step the pH was carefully maintained between 9 and 10 by addition of NaOH 10N. No further changes indicated that the coupling reaction was complete. The derivatized Sepharose 4B, throughly washed with distilled water and equilibrated with 3mM phosphate buffer pH 6.8, was used for the purification procedure.

1. Collection of plasma

A volume of 10 L of bovine blood was collected at the slaughterhouse, mixed with 1 L of 2.5 % sodium citrate solution and centrifuged at 3000 g for 20 min, retaining the plasma.

2. Ammonium Sulphate Fractionated Precipitation.

Ammonium sulphate was added to 4 L of plasma to 35% of saturation, stirred for 2 hrs at 4°C and centrifuged at 10 000 g for 20 min, retaining the supernatant to which ammonium sulphate has been added up to 55% saturation, maintaining the stirring for 30 min at 4°C and then centrifuging at 10 000 g for 20 min. The precipitate was retained and dissolved in 200 mL of 0.1 M potassium phosphate buffer, pH 7.2. The solution was dialysed for 20 hrs against 20 L of a 10 mM potassium phosphate buffer, pH 7.2 with two changes of buffer.

The dialysed plasma proteins solution was centrifuged at 10

000 g for 20 min and the precipitate was discarded.

3. AE-Agarose Chromatography and CP Purification.

The AE-Agarose column (30 x 2.5 cm) was equilibrated with a 10 mM potassium phosphate buffer, pH 7.2, which was also used as first eluant. The dialysed plasma protein solution was applied onto the AE-Agarose column, at a flow rate of 120 mL/h. Preferably the fraction that passed through the column was collected for further BSAO purification. The AE-Agarose column was then washed with: 500 ml of starting 10 mM phosphate buffer, then with 200 mL of 20 mM buffer solution and finally with 100 mL of 30 mM phosphate buffer, all at pH 7.2. The retained CP was eluted with 100 mL of a 0.2 M phosphate buffer, collecting (Pharmacia, LKB: Frac-200) fractions of 2 mL each. The fractions with the ratio A610/A280≥0.05 were collected in a pool with a CP concentration of 5 - 10 mg/mL.

Being fast and highly selective , this method minimizes the ceruloplasmin proteolytic degradation, ensuring thus a particularly high molecular integrity and specific activity. For comparative studies we retained ceruloplasmin with different degrees of molecular integrity (A610/A280 = 0.02; 0.04; 0.06)

Purified CP was stored at -20°C.

Ultrafiltration

If higher CP concentrations are required than those obtained directly from the column, a further ultrafiltration step (Amicon 8200, membrane YM 100) leading to a final purified CP at a concentration of about 20 mg/mL.

Ceruloplasmin enzyme activity may be determined following the

Osaki et al.'s method(Di Pietro D, Tavazzi B, Lazzarino G, Giardina B. Malondialdehyde is a biochemical marker of peroxidative damage in the isolated reperfused rat heart. Mol Cell Biochem;1992;116:193-96) with p-phenylenediamine as substrate. One enzyme unit is considered the amount of enzyme that would generate an increase of one absorbancy unit/min at λ=540 nm.

The protein concentrations were determined by the Bradford (Bolli J, Jeroudi MO, Patel BS. Marked reduction of free radical generation and contractile dysfunction by anti-oxidant therapy begun at the time of reperfusion; evidence that myocardial "stunning" is a manifestation of reperfusion injury. Circ Res 1989;65:607-22.) method.

Electrophoresis

The degree of homogeneity as well as the molecular integrity and characteristics of purified CP were evaluated by SDS Fast Electrophoresis (Pharmacia-LKB: Phast System), in the presence of 5 % β-mercaptoethanol. Onlyone band characteristic for homogeneous ceruloplasmin (132 Kda) has been obtained.

The aforesaid modified method described (with ammonium sulphate precipitation) yields larger amounts of purified CP protein.

The characteristics of CP purified by the modified method including ammonium sulphate precipitation, are better than that those obtained by other procedure. For instance, the specific activity is high (0.70 EU/mg) and the purity expressed by the spectral parameters (A610/A280) is excellent (0.062) when compared with data obtained with other methods.

Example 2

Dose-effect relationship of the antifibrillatory action of Ceruloplasmin on isolated heart in arrhythmias.

Isolated rat heart preparation

All experiments conforms with the Guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication No 85-23, revised 1985).
Adult male Wistar rats (225-250 g) were anaesthetised with sodium pentobarbitone (0.1 mL/100 g body weight) and then heparinised intravenously (500 UI). Hearts were rapidly excised, placed in ice-cold oxygenated Krebs-Henseleit (KH) buffer solution and then mounted on a modified Langendorff heart perfusion apparatus.

Hearts were cannulated via the aorta and retrogradely perfused at a constant perfusion pressure (80 mm Hg at 37 0C) with modified KH buffer containing the following (mM/L): NaCl, 120; NaHCO3 25.4; KCl, 3.8; KH2PO4 1.2; MgSO4, 0.86; CaCl2, 1.25 and glucose, 11. This solution was continuouslly gassed with a mixture of 95% O2 and 5% CO2 to maintain a pH of 7.4 at 37 íC. In order to avoid the precipitation of calcium, the perfusion buffer was filtered through a 5.0 μm cellulose acetate membrane to remove particulate contaminants.

Recorded cardiodynamic indices

A saline-filled latex ballon was inserted into the left ventricle by way of the AV valve and connected to a pressure transducer for determination of Left Ventricular Pressure (LVP) and Left Ventricular End Diastolic Pressure (LVEDP). Epicardial electrogram (ECG) was obtained using two silver electrodes, one inserted into the ventricular apex,and the other connected to the aortic cannula. The LVP, LVEDP, and ECG were recorded on a Nihon-Kohden polygraph (RM 600); heart

rate was calculated from the electrogram. Coronary flow (CF) was measured by time collection of coronary effluent at various times during the experiment.

Experimental design

Hearts were perfused for a 20 min control period with KH buffer. Regional ischaemia was induced by occluding the left anterior descending artery with a ligature positioned around and at a point close to its origin. The resulting arterial occlusion that produces a reduction in coronary flow of 40% - 50%, was maintained for 15 min. At the end of this period, reperfusion was achieved by cutting the ligature and rhythm disturbances were monitored for 10 min.

Ten experimental groups were studied. Hearts in the control group (n = 12) were perfused with KH buffer throughout the experiment and submitted to 15 min partial ischemia without ceruloplasmin treatment.

For the dose-effect relationship study, ischemic hearts (n = 8) were treated with KH perfusing buffer containing different concentrations of ceruloplasmin (0.25 μM, 0.50 μM, 1 μM, 2 μM) with optimal degree of molecular integrity (A610/A280 = 0.06), 5 min before reperfusion.

Quantification of arrhythmias

Arrhythmias were defined according to the Lambeth conventions. Electrogram recordings were analyzed for 1) number of premature ventricular beats (VPBs); 2) incidence of ventricular tachycardia (VT), defined as a run of four or more consecutive VPBs; 3) incidence of total ventricular fibrillation (including both reversible and irreversible ventricular fibrillations) and 4) incidence of irreversible ventricular fibrillations. It was analyzed whether

fibrillation was spontaneously reversible, or hearts remained in irreversible ventricular fibrillation (more than 120 seconds).

Ventricular fibrillation was defined as a ventricular rhythm with no recognizable QRS complex and with an amplitude less than that of the normal electrogram. In addition, the total time during which each heart remained in normal sinus rhythm during the first 5 minutes of reperfusion, was quantified.

Statistical analysis

Statistical significance of differences in incidence of total and irreversible ventricular fibrillations and ventricular tachycardia between the treated and control groups was evaluated with a Fisher's exact test (37). With the exceptions of incidences of arrhythmias, all the results are expressed as mean (+ SEM). A significant difference was taken for (P) values less than 0.05 ($P < 0.05$).

Effect of ceruloplasmin on reperfusion arrhythmias

The dose-related cardioprotection afforded by ceruloplasmin is presented in Table I. Control hearts exhibited 100% total ventricular fibrillations (VF) after 15 minutes of partial ischemia and for 83% of them, fibrillation was irreversible (more than 120 seconds). The incidence of ventricular tachycardia (VT) was 100% and the total duration of normal sinus rhythm during 5 minutes of reperfusion was extremely short, only 25 sec.

Ceruloplasmin significantly reduced the incidence of reperfusion-induced total ventricular fibrillations from 100% to 42% for dose of 1μM and to 25% for the dose of 2 μM (Table I). More important, the incidence of irreversible ventricular fibrillations decreased from 83% to 0%.

Associated with the decrease in incidence and duration of ventricular fibrillations, a large increase in the total duration of normal sinus rhythm was observed, in a concentration dependent manner, during the 5 minutes of reperfusion from 25 sec to 248 sec (Table I).

Example 3

The effects of protein molecular integrity on the antiarrhythmic properties of ceruloplasmin.

The isolated heart system was prepared and measurements were carried out as described in the example 1.
The role of Ceruloplasmin molecular integrity on cardioprotection was studied on ischemic hearts (n = 8) perfused with KH buffer containing ceruloplasmin (1 μM) at three different degrees of molecular integrity (A610/A280 = 0.02; 0.04; 0.06), 5 min before reperfusion.

The incidence of total ventricular fibrillation decreased from 100% to 42% and that of irreversible ventricular fibrillation from 87% to 0% under treatment with 1μM ceruloplasmin exhibiting an increased ratio A610/A280 from 0.02 to 0.06 (Table II). The duration of normal sinus rhythm also increased as a function of the degree of ceruloplasmin molecular integrity.

Effect of heat denatured ceruloplasmin

Heat denaturation (boiling) inactivates the ceruloplasmin enzyme activity, with loss of the blue color and of the integrity of the blue-copper center (A610 = 0). The results clearly indicated that denatured ceruloplasmin has no protective effects on reperfusion-induced arrhythmias and on the total duration of normal sinus rhythm during the first 5 minutes of reperfusion period.

Ceruloplasmin molecular integrity is therefore an important factor for its ability as OFR scavenger

Effect of deferoxamine

Deferoxamine administered 5 min before coronary occlusion reduced the incidence of total ventricular fibrillation from 100% to 50% while ceruloplasmin (1μM), reduced the incidence from 100% to 33%, showing a better protection (Table III) and acting at a much lower concentration (1μM vs 500 μM).

The embodiments of the invention in which an exclusive property or priviledge is claimed, are defined as follows:

1. Use of ceruloplasmin or pharmaceutically acceptable derivative of ceruloplasmin, as antifibrillatory agent against heart arrhythmias.

2. Use according to claim 1, wherein the ceruloplasmin shows a spectral parameters ratio ($Absorption_{610}/Absorption_{280}$) greater than 0.04.

3. Use according to claim 1, wherein the ceruloplasmin shows a spectral parameters ratio ($Absorption_{610}/Absorption_{280}$) of at least 0.06.

4. Use according to claim 1, wherein the ceruloplasmin is obtained by a process involving the following sequence of essential steps:

- fractional precipitation of plasma proteins;
- recovering of the supernatant;
- adsorbing the proteins still contained in the supernatant on a chromatographic support comprising an aminoethyl resin; and
- desorbing the proteins adsorbed on the chromatographic support with an appropriate eluant and recovering a chromatographic fraction containing ceruloplasmin having a spectral parameters ratio ($Absorption_{610}/Absorption_{280}$) greater than 0.04.

5. Use according to claim 4, wherein the ceruloplasmin has a spectral parameters ratio ($Absorption_{610}/Absorption_{280}$) of at least 0.06.

6. Use according to claim 4, wherein the fraction of protein containing the ceruloplasmin having a spectral parameters ratio ($Absorption_{610}/Absorption_{280}$) greater than

0.04, is further associated with a pharmaceutically acceptable excipient to define a pharmaceutically acceptable composition.

7. Use according to claim 4, wherein the fraction of protein containing the ceruloplasmin having a spectral parameters ratio ($Absorption_{610}/Absorption_{280}$) of at least 0.06, is further associated with a pharmaceutically acceptable excipient to define a pharmaceutically acceptable composition.

8. Use according to claim 6, wherein the pharmaceutically acceptable excipient is of the type commonly used for the preparation of a composition for an intravenous injection.

9. Use according to claim 6, wherein the composition has a ceruloplasmin concentration of at least 0.50μM.

10. Use according to claim 6, wherein the composition has a ceruloplasmin concentration of at least 1μM.

11. An antifibrillatory pharmaceutical composition against heart arrhythmias, wherein it comprises in association with a pharmaceutically acceptable carrier, a ceruloplasmin, pharmaceutically acceptable derivative of ceruloplasmin.

12. An antifibrillatory composition according to claim 11, wherein the ceruloplasmin shows a spectral parameters ratio ($Absorption_{610}/Absorption_{280}$) greater than 0.04.

13. An antifibrillatory composition according to claim 11, wherein the ceruloplasmin shows a spectral parameters ratio ($Absorption_{610}/Absorption_{280}$) of at least 0.06.

14. An antifibrillatory composition according to claim 11, wherein the ceruloplasmin is obtained by a process involving

the following sequence of essential steps:

- fractional precipitation of plasma proteins;
- recovering of the supernatant;
- adsorbing the proteins still contained in the supernatant on a chromatographic support comprising an aminoethyl resin; and
- desorbing the proteins adsorbed on the chromatographic support with an appropriate eluant and recovering a chromatographic fraction containing ceruloplasmin having a spectral parameters ratio ($Absorption_{610}/Absorption_{280}$) greater than 0.04.

15. An antifibrillatory composition acording to claim 14, wherein the ceruloplasmin has a spectral parameters ratio ($Absorption_{610}/Absorption_{280}$) of at least 0.06.

16. Method for treating or preventing heart arrhythmias wherein a composition as defined in claim 11 is administered to said heart.

17. Method for treating or preventing heart arrhythmias wherein a composition as defined in claim 12 is administered to said heart.

18. Method for treating or preventing heart arrhythmias wherein a composition as defined in claim 13 is administered to said heart.

19. Method for treating or preventing heart arrhythmias wherein a composition as defined in claim 14 is administered to said heart.

20. Method for treating or preventing heart arrhythmias wherein a composition as defined in claim 15 is administered to said heart.

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(54) Title: ANTIFIBRILLATORY AGENT IN MYOCARDIAL REPERFUSION

(57) Abstract

The invention relates to a new use of a known chemical compound as a therapeutic agent having antiarrhythmic properties, the ceruloplasmin (a copper protein). More particularly, the Applicants have noted that ceruloplasmin generates antiarrhythmic effects during reperfusion in the ischemic heart. The invention also relates to a method for either preventing or treating heart arrhythmias.

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FASEB JOURNAL FOR EXPERIMENTAL BIOLOGY, vol. 8, no. 5, 18 March 1994 BETHESDA, MD US, page A606 ATANASIU R. ET AL. 'Antiarrhythmic effects of ceruloplasmin as free radical scavenger in ischaemic isolated rat heart' see abstact 3512 ---	1-20
A	J. SURG. RES. (1991), 51(1), 60-5 CODEN: JSGRA2;ISSN: 0022-4804, 1991 BARON, PEDRO ET AL 'Renal preservation after warm ischemia using oxygen free radical scavengers to prevent reperfusion injury' see the whole document ---	1-20
	-/--	

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 September 1995	16. 11. 95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Authorized officer Moreau, J

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PREP. BIOCHEM. (1994), 24(3&4), 237-50 CODEN: PRBCBQ;ISSN: 0032-7484, 1994 WANG, X. T. ET AL 'Joint chromatographic purification of bovine serum ceruloplasmin and amine oxidase' see the whole document ---	2-4,14
X	CAN. J. PHYSIOL. PHARMACOL. (1991), 69(10), 1459-64 CODEN: CJPPA3;ISSN: 0008-4212, 1991 CHAHINE, RAMEZ ET AL 'Protective effects of ceruloplasmin against electrolysis-induced oxygen free radicals in rat heart' see the whole document ---	2-4,14
O,X	DAS, D. K. (ED.). ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, VOL. 723. CELLULAR, BIOCHEMICAL, AND MOLECULAR ASPECTS OF REPERFUSION INJURY;CONFERENCE, NEW YORK, NEW YORK, USA, JULY 11-14, 1993. XVI+506P. NEW YORK ACADEMY OF SCIENCES: NEW YORK, NEW YO, BARON P ET AL 'Ceruloplasmin and deferoxamine prevent ischemia- reperfusion damage in kidney transplantation.' see the whole document ---	1-20
P,X	ARZNEIM.-FORSCH. (1995), 45(4), 476-80 CODEN: ARZNAD;ISSN: 0004-4172, 1995 MATEESCU, M. A. ET AL 'Protection of myocardial tissue against deleterious effects of oxygen free radicals by ceruloplasmin' see the whole document -----	1-20

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA95/00232

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: although claims 1-10, 16-20 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.